Activation of a histone H1 kinase by tyrosine phosphorylation in v-src-transformed fibroblasts

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Using anti-phosphotyrosine immunoaffinity chromatography, we have searched for serine/threonine kinases that are directly regulated by tyrosine phosphorylation in v-src-transformed rat 3Y1 fibroblasts. Tyrosine phosphoprotein preparations from v-src-transformed cells contain a kinase activity that phosphorylates histone H1 *in vitro* on serine residues and this activity is present at a 20-fold greater level than that in parental cell preparations. This activity elutes from a MonoQ FPLC column as a single peak and gel filtration chromatography suggests that the kinase has a molecular mass of ~55 kDa. Tyrosine phosphatase treatment inactivates the histone H1 kinase and this result indicates that the specific activity of the kinase is regulated by tyrosine phosphorylation. Experiments with cells transformed with a temperature-sensitive mutant of the v-src oncogene demonstrate that the tyrosine phosphorylation of the histone H1 kinase is an early event in v-src transformation. The kinase is distinct from known cdc2 family members that contain the PSTAIR motif, because the kinase can be separated almost completely from these proteins by immunoprecipitation with an antibody against p34^{cdc2}. The profile of antibody reactivity and sensitivity to modulators of protein kinases suggests that this activity is distinct from known second messenger-regulated kinases and from previously characterized MAP kinases.

Key words: cell transformation/intracellular signaling/serine kinase/tyrosine phosphorylation/v-src

Introduction

Expression of v-src, which encodes the membrane-associated phosphoprotein pp60^{v-src}, mediates the transformation of fibroblasts infected with Rous sarcoma virus (for recent reviews see Jove and Hanafusa, 1987; Golden and Brugge, 1988; Parsons and Weber, 1989). pp60^{v-src} possesses an inherent protein tyrosine kinase activity (Collett *et al.*, 1980; Hunter and Sefton, 1980; Levinson *et al.*, 1980) that is presumed to be essential for transformation (Sefton *et al.*, 1980; Iba *et al.*, 1985). Many early cellular responses are elicited by transformation of fibroblasts with the v-src oncogene. Tyrosine phosphorylation of the fibronectin receptor (Hirst *et al.*, 1986), calmodulin (Fukami *et al.*, 1986), vinculin (Sefton *et al.*, 1981), ezrin (Gould *et al.*,

1986), talin (Pasquale *et al.*, 1986), the calpactin I heavy chain (Radke *et al.*, 1980), paxillin (Glennay and Zokas, 1989; Turner *et al.*, 1990) and numerous other substrates has been demonstrated in v-src-transformed cells. The p21^{ras} GTPase-activating protein (GAP) is tyrosine-phosphorylated in v-src-transformed cells (Ellis *et al.*, 1990) and complexes with pp60^{v-src} (Brott *et al.*, 1991). GAP negatively regulates Ras proteins by stimulating their intrinsic GTPase activities (reviewed in McCormick, 1989) and this tyrosine phosphorylation of GAP might modify Ras-dependent signaling. pp60^{v-src} complexes with PI-3 kinase activity (Fukui and Hanafusa, 1989) and this activity is elevated in v-src-transformed fibroblasts (Fukui *et al.*, 1991). Although the mechanism of this PI-3 kinase activation has not yet been elucidated, evidence for the phosphorylation of this enzyme on tyrosine residues has been provided (Fukui and Hanafusa, 1991).

Both growth factor receptor and non-receptor-type tyrosine kinases have been shown to initiate the activation of serine/threonine kinases (for reviews see Czech *et al.*, 1988; Avruch *et al.*, 1990; Weiel *et al.*, 1990). Expression of pp60^{v-src} in chicken embryo fibroblasts (CEF) increases the activity of the pp70 ribosomal protein S6 kinase (Blenis and Erikson, 1985; Blenis *et al.*, 1987) and pp60^{v-src} increases pp90 S6 kinase II activity when coexpressed in recombinant baculovirus-infected insect cells (Vik *et al.*, 1990). However, activated pp70 S6 kinase and pp90 S6 kinase II isolated from mitogen-stimulated cells have shown only serine and threonine phosphorylation, suggesting that these kinases are not regulated directly by tyrosine phosphorylation (Ballou *et al.*, 1988; Erikson and Maller, 1989; Sweet *et al.*, 1990). MAP kinase from insulin-stimulated cells phosphorylates and activates the pp90 S6 kinase II *in vitro* (Sturgill *et al.*, 1988). A MAP kinase activator (termed MAP kinase kinase) has been reported (Ahn *et al.*, 1991; Gómez and Cohen, 1991) and this activity phosphorylates the p42 isoform of MAP kinase on tyrosine and threonine residues (L'Allemain *et al.*, 1992; Nakielnny *et al.*, 1992). The p42 MAP kinase is constitutively activated in v-src-transformed cells (Gupta *et al.*, 1992), but the role of MAP kinase kinase in this signaling cascade has not yet been clarified. Recent findings have suggested that signaling by pp60^{v-src} is mediated by protein kinase C (Spangler *et al.*, 1989; Nori *et al.*, 1990; Qureshi *et al.*, 1991). Transformation of fibroblasts with v-src has been reported to increase Raf-1 protein tyrosine phosphorylation and associated serine/threonine kinase activity (Morrison *et al.*, 1988). Association of pp60^{v-src} with a Ca²⁺/calmodulin-dependent protein kinase activity has also been reported (David-Pfeuty and Nouvian-Dooghe, 1990). These findings suggest that transformation by v-src activates numerous serine/threonine kinases through a cascade of sequential protein phosphorylations.

Little is known about the initiation of this signaling cascade by pp60^{v-src} and we have therefore searched for serine/threonine kinases that are directly activated by tyrosine

phosphorylation in *v-src*-transformed fibroblasts. We present a novel application of anti-phosphotyrosine immunoaffinity chromatography to recover serine/threonine kinase activity

Fig. 1. Tyrosine phosphoprotein and histone H1 kinase binding to PY20. Serum-starved 3Y1 or SR-3Y1 cell lysates were bound to PY20-Affi-Gel and the resin was packed into a column and washed extensively. A final 2 column vol wash fraction was collected and bound protein was eluted with sequential 2 column vol fractions of 40 mM phenyl phosphate. **A.** Column eluates were separated by SDS-PAGE (10%), transferred to a nylon membrane and probed with polyclonal antiserum against phosphotyrosine. **B.** Column eluates were subjected to *in vitro* histone H1 kinase assay and reaction mixtures were separated by SDS-PAGE (12.5%). Molecular weight standards (kDa) are shown at left and arrow indicates the position of histone H1.

in tyrosine phosphoprotein preparations. Monoclonal antibodies directed against phosphotyrosine were used previously to purify large amounts of tyrosine phosphoprotein by immunoaffinity chromatography (Glenney and Zokas, 1989; Kanner *et al.*, 1989) and these enriched preparations were used to generate monoclonal antibodies against individual tyrosine phosphoproteins (Glenney and Zokas, 1989; Kanner *et al.*, 1990). Using a similar strategy, we have identified a kinase present in tyrosine phosphoprotein preparations from *v-src*-transformed fibroblasts that phosphorylates histone H1 *in vitro* on serine residues. This kinase is stimulated early in *v-src* transformation and the activation requires tyrosine phosphorylation of the kinase.

Results

A histone H1 kinase activity is present in tyrosine phosphoprotein preparations from SR-3Y1 Cells

In order to enrich for serine/threonine kinases phosphorylated on tyrosine in *v-src*-transformed fibroblasts, cell lysates were incubated with an anti-phosphotyrosine immunoaffinity resin. PY20, a monoclonal antibody directed against phosphotyrosine residues (Glenney *et al.*, 1988), was used in this enrichment. The properties of this antibody were compared with those of a polyclonal antiserum against phosphotyrosine (anti-PTYR) (Hamaguchi *et al.*, 1988), which has been shown to precipitate >90% of tyrosine phosphoproteins from *v-src*-transformed fibroblasts. By immunoprecipitation and immunoblotting, the PY20 antibody was found to recognize a population of tyrosine phosphoproteins similar to that recognized by anti-PTYR antibody (unpublished data), indicating that the PY20 antibody can bind to a wide range of tyrosine kinase substrates. After incubation of cell lysates with PY20 antibody coupled to Affi-Gel, bound phosphoproteins were eluted with 40 mM phenyl phosphate. As shown in Figure 1A, numerous tyrosine phosphoproteins from *v-src*-transformed 3Y1 cells (SR-3Y1) were bound to and eluted from the PY20 resin. The majority of tyrosine phosphoprotein was present in the first phenyl phosphate

Fig. 2. MonoQ FPLC fractionation of PY20 eluate and demonstration of serine kinase activity. 3Y1 and SR-3Y1 PY20 eluates were fractionated by MonoQ FPLC at pH 7.2. **A.** PY20 eluate was applied to the column and after a 4.0 ml wash with 50 mM NaCl (1.0 ml/min), the column was developed with a linear gradient of NaCl. Fractions were assayed for histone H1 kinase activity. Arrow indicates the position of histone H1. Molecular weight standards (kDa) are shown at right. **B.** SR-3Y1 PY20/MonoQ fractions 9–12 were pooled and used to phosphorylate histone H1. The ^{32}P -labeled histone H1 was subjected to phosphoamino acid analysis. Positions of inorganic phosphate, phosphoserine, phosphothreonine and phosphotyrosine are shown.

fraction. With the exception of a background signal at 44 kDa, which was presumably due to non-specific reactivity of ovalbumin carrier protein present in collection tubes, tyrosine phosphoproteins were not detected in PY20 eluates derived from parental 3Y1 cells.

The presence of serine/threonine kinase activity in the PY20 eluates was determined by assaying the phosphorylation of exogenously added substrate. Protein kinase activities phosphorylating multiple substrates, including MAP-2, myelin basic protein, casein, histone H1 and protamine, were elevated in PY20 eluates derived from SR-3Y1 cells relative to those from parental 3Y1 cells (Figure 1B and unpublished data). The histone H1 kinase activity in SR-3Y1 PY20 eluates showed the greatest relative increase, typically >20-fold more than that present in 3Y1 PY20 eluates (Figure 1B). Consequently, this activity was further characterized. Similar to the rapid elution of tyrosine phosphoproteins, >75% of the histone H1 kinase activity was recovered in the first phenyl phosphate fraction. The phosphorylation of histone H1 was linear with respect to time for >20 min (unpublished data). The phosphorylation of multiple endogenous substrates was also observed. The recognition of a histone H1 kinase activity was not unique to the PY20 antibody, because anti-PTYR and the monoclonal anti-phosphotyrosine antibody 4G10 (Drucker *et al.*, 1989) precipitated elevated histone H1 kinase activity from SR-3Y1 cell lysates compared with that from control 3Y1 lysates (unpublished data).

The PY20 eluates were fractionated by anion exchange chromatography using MonoQ FPLC. Column fractions were subjected to *in vitro* kinase assay using histone H1 as the substrate. As shown in Figure 2A, the histone H1 kinase activity of SR-3Y1 PY20 column eluates was recovered as a single peak from the MonoQ column. The activity eluted from the MonoQ column at 360–420 mM NaCl. No histone H1 kinase activity was detected elsewhere in the column profile (unpublished data). Negligible histone H1 kinase activity was observed when a PY20 eluate derived from 3Y1 cells was applied to the MonoQ column (Figure 2A).

Histone H1 kinase is a serine kinase regulated by tyrosine phosphorylation

To determine whether a serine/threonine kinase was responsible for the observed histone H1 phosphorylation, phosphoamino acid analysis was performed on *in vitro* phosphorylated histone H1. MonoQ column fractions 9–12 derived from the SR-3Y1 PY20 eluate (Figure 2A) were pooled and histone H1 was phosphorylated using this kinase preparation. As shown in Figure 2B, phosphoamino acid analysis of the histone H1 substrate showed phosphorylation of serine residues. Similar results were obtained when histone H1 phosphorylated by unfractionated SR-3Y1 PY20 eluate was subjected to phosphoamino acid analysis (unpublished data).

In order to assess the molecular weight of this histone H1 kinase, PY20 eluates from SR-3Y1 cells were fractionated by Superose 12 gel filtration chromatography (Figure 3). A single peak of histone H1 kinase activity eluted from the Superose 12 column and comparison of its elution position with that of protein standards suggested that the kinase has a molecular mass of 55 ± 5 kDa.

The increased recovery of histone H1 kinase activity in SR-3Y1 PY20 eluates relative to 3Y1 PY20 eluates could

potentially be due to adventitious tyrosine phosphorylation of a serine kinase without an effect on specific activity. Adventitious tyrosine phosphorylation could increase the binding of a histone H1 kinase to the PY20 resin and increased kinase activity would therefore be recovered in the eluate. Alternatively, tyrosine phosphorylation of the histone H1 kinase may alter its specific activity. To determine whether the specific activity of the histone H1 kinase is modulated by tyrosine phosphorylation, a PY20 eluate derived from SR-3Y1 cells was treated *in vitro* with catalytic domain 1 of the leukocyte antigen related (LAR) protein, which has been shown to have an intrinsic tyrosine phosphatase activity (Streuli *et al.*, 1990). This LAR fragment is at least 10^4 -fold more specific for phosphotyrosine hydrolysis compared with phosphoserine or phosphothreonine hydrolysis (Cho *et al.*, 1991). As shown in Figure 4, LAR treatment of the PY20 eluate decreased the histone H1 kinase activity by >5-fold. Incubation in the

Fig. 3. Gel filtration chromatography of SR-3Y1 PY20 eluate. SR-3Y1 PY20 eluate was applied to a Superose 12 gel filtration column. The column was developed at a flow rate of 1.0 ml/h and 0.25 ml fractions were collected. Column fractions were assayed *in vitro* for histone H1 kinase activity. Position of histone H1 is indicated by arrows. Elution positions of molecular weight standards are shown above.

Fig. 4. Inactivation of histone H1 kinase with LAR tyrosine phosphatase. SR-3Y1 fraction 1 PY20 eluate was incubated in the presence or absence of catalytic domain 1 of the LAR tyrosine phosphatase. Histone H1 kinase assay was then performed and the reaction mixtures were separated by SDS-PAGE (12.5%). Lane 1, PY20 eluate was incubated in the absence of LAR; lane 2, PY20 eluate was incubated with LAR and $\text{Na}_3\text{VO}_4/\text{Na}_2\text{MoO}_4$ were added prior to kinase assay; lane 3, PY20 eluate was incubated with LAR in the presence of $\text{Na}_3\text{VO}_4/\text{Na}_2\text{MoO}_4$; lane 4, PY20 eluate was incubated in the absence of LAR and $\text{Na}_3\text{VO}_4/\text{Na}_2\text{MoO}_4$ was added prior to histone H1 kinase assay. Molecular weight standards (kDa) are shown at left and arrow indicates the position of histone H1.

presence of the tyrosine phosphatase inhibitors Na_3VO_4 and Na_2MoO_4 fully protected the histone H1 kinase activity during the phosphatase treatment. Na_3VO_4 and Na_2MoO_4 alone had only a slight inhibitory effect on the observed kinase activity. These results indicated that tyrosine phosphorylation modulates the specific activity of the histone H1 kinase in *v-src*-transformed fibroblasts.

The 3Y1 and SR-3Y1 cell lines have been passaged extensively and it remained possible that the differences in histone H1 kinase activity were due to the accumulation of independent mutations in the genomes rather than to transformation by the *v-src* oncogene. To address this point, a cell line (3Y1-NY72^{ts}) transformed with a temperature-sensitive mutant of *v-src* was used. Cells were maintained at a temperature permissive (34°C) or non-permissive (39.5°C) for transformation and PY20 eluates were prepared from cell lysates. As shown in Figure 5A, the histone H1 kinase activity present in the PY20 eluate from cells maintained at the permissive temperature was 5-fold greater than that in the PY20 eluate from cells maintained at the non-permissive temperature. The histone H1 kinase activities recovered from 3Y1 cells maintained at either temperature were identical; the incubation temperature also had no effect on the activity recovered from SR-3Y1 cells (unpublished data). These results demonstrated that even in a uniform genetic background, *v-src* transformation is associated with the tyrosine phosphorylation of a histone H1 kinase.

Histone H1 kinase tyrosine phosphorylation occurs early in *v-src* transformation

To determine whether tyrosine phosphorylation of the histone H1 kinase is an early event in *v-src* transformation, the 3Y1-NY72^{ts} cell line was briefly shifted from the non-permissive to the permissive temperature. After 15 min at the permissive temperature, cells were lysed and PY20 eluates prepared. As shown in Figure 5B, the histone H1 kinase activity in PY20 eluates was increased by >3-fold after this brief shift to the permissive temperature. Immunoprecipitation of cell lysates with polyclonal anti-phosphotyrosine antibody followed by immune complex kinase assay showed increased histone H1 kinase activity within 5 min of temperature shift (unpublished data), providing further evidence that the tyrosine phosphorylation and activation of the histone H1 kinase occurs early in *v-src* transformation.

The histone H1 kinase is distinct from second messenger-regulated kinases, *p34^{cdc2}* and MAP kinases

A systematic search was conducted to identify the histone H1 kinase activity present in PY20 eluates. The kinase activity was not appreciably affected by Ca^{2+} , Ca^{2+} /calmodulin, bromo-cAMP or bromo-cGMP (Table I). Consistent with these findings, inhibitory peptides targeting cAMP-dependent protein kinase (JK4 peptide), protein kinase C (JK77 peptide) and Ca^{2+} /calmodulin-dependent protein kinase (BB91 peptide) had little effect on the observed histone H1 kinase activity. Each peptide inhibitor was included in the histone H1 kinase reaction at a concentration at least 10-fold higher than the K_i for the respective target enzyme (Cheng *et al.*, 1986; House and Kemp, 1987; Nichols *et al.*, 1990). These results indicated that the histone H1 kinase activity is unlikely to be attributable to protein kinase C, Ca^{2+} /calmodulin-dependent protein kinase,

Fig. 5. PY20 eluates from cells transformed with a temperature-sensitive mutant of *v-src*. **A.** 3Y1-NY72^{ts} cells were maintained at a temperature permissive (34°C) or non-permissive (39.5°C) for transformation. **B.** 3Y1-NY72^{ts} cells were maintained at 39.5°C or shifted from 39.5°C to 34°C for 15 min. PY20 eluates were prepared as in Figure 1 and *in vitro* histone H1 kinase assay was performed. Molecular weight standards (kDa) are shown at left and arrow indicates the position of histone H1.

Table I. Effect of kinase modifiers on histone H1 kinase activity

Modifier	Histone H1 kinase activity (percent of control) ^a
No addition	100%
Ca^{2+} (2 mM) ^b	87%
Ca^{2+} /Calmodulin (1.7 mM/ 17 $\mu\text{g/ml}$) ^b	74%
8-bromo-cAMP (10 μM)	106%
8-bromo-cGMP (10 μM)	94%
JK4 peptide (25 nM)	83%
JK77 peptide (1.5 μM)	79%
BB91 peptide (30 μM)	75%
Staurosporine (200 nM)	15%

^aA value of 100% is equivalent to an enzyme activity to incorporate 10 fmol of phosphate/min/ml of PY20 eluate.

^bSamples and controls were assayed in the absence of EGTA.

cAMP-dependent protein kinase or cGMP-dependent protein kinase (reviewed in Edelman *et al.*, 1987).

p34^{cdc2} is a histone H1 kinase that is phosphorylated on threonine and tyrosine residues during G₂ phase of the cell cycle (Draetta *et al.*, 1988; Morla *et al.*, 1989). The relationship between the histone H1 kinase present in SR-3Y1 PY20 eluates and *p34^{cdc2}* was determined. As shown in Figure 6A, *p13^{suc1}* coupled to Sepharose beads could precipitate histone H1 kinase activity. *p13^{suc1}* has been shown to complex with members of the *cdc2* family of protein kinases (Brizuela *et al.*, 1987; Arion *et al.*, 1988; Dunphy *et al.*, 1988; Fang and Newport, 1991). However, the *p13^{suc1}* protein is known to complex non-specifically with numerous other proteins and the identity of the histone H1 kinase was elucidated by direct immunoprecipitation of 3Y1 and SR-3Y1 PY20 eluates with a polyclonal antibody (G8 antibody) against *p34^{cdc2}*. The histone H1 kinase activity was almost exclusively retained in the supernatant

Fig. 6. p13^{suc1} and G8 antibody precipitation of PY20 eluates. **A.** 3Y1 or SR-3Y1 PY20 eluates were incubated with p13^{suc1}- or mock-coupled Sepharose beads (lanes 1–4). Beads were washed and *in vitro* histone H1 kinase assay was performed. Lane 5, p13^{suc1}-coupled beads incubated in the absence of PY20 eluate. **B.** Equivalent fractions of 3Y1 or SR-3Y1 PY20 eluates, PY20 eluates immunoprecipitated with G8 antibody, or the supernatants of PY20 eluates after G8 precipitation were assayed *in vitro* for histone H1 kinase activity. **C.** PY20 eluates, G8 immunoprecipitates or supernatants after G8 precipitation were immunoblotted with anti-PSTAIR antibody. Molecular weight standards (kDa) are shown at left and arrow indicates the position of histone H1.

(Figure 6B). Incubation of PY20 eluate with G8 antibody had no effect on histone H1 kinase activity in solution (unpublished data). The p34^{cdc2} protein contains a conserved sequence, EGVSTAIRISLLKE, which has been termed the PSTAIR motif; this sequence is present in all known homologs of p34^{cdc2} from yeast to humans (Lee and Nurse, 1987; reviewed in Draetta, 1990). To monitor the partitioning of p34^{cdc2}, PY20 eluates immunoprecipitated with G8 antibody were immunoblotted with anti-PSTAIR antibody (Figure 6C). An anti-PSTAIR-reactive 34 kDa protein was almost completely immunoprecipitated by G8 antibody. Therefore, the separation of anti-PSTAIR-reactive protein from the observed histone H1 kinase indicates that the activity is distinct from the *cdc2* family of protein kinases. The 34 kDa protein recognized by the anti-PSTAIR antibody was not associated with histone H1 kinase activity. Dephosphorylation of p34^{cdc2} on tyrosine and threonine residues is correlated with an increase in histone H1 kinase activity (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Morla *et al.*, 1989). Therefore, the tyrosine-phosphorylated p34^{cdc2} that is present in PY20 eluates might be relatively inactive.

Fig. 7. MonoQ fractionation of histone H1 kinase activity and MAP kinase. SR-3Y1 whole cell lysate was applied to the column and after a 10 ml wash with 50 mM NaCl (0.5 ml/min), the column was developed with a linear gradient of NaCl. **A.** Fractions were immunoprecipitated with PY20 beads and assayed for histone H1 kinase activity. Arrows indicate position of histone H1. **B.** Fractions were immunoblotted with antibody against the p42 and p44 isoforms of MAP kinase. Molecular weight standards are shown at left.

The relationship between MAP kinase and the observed histone H1 kinase was determined by using a mouse monoclonal antibody that recognizes the p42 and p44 isoforms of MAP kinase. A SR-3Y1 whole cell lysate was applied to a MonoQ FPLC column and eluted fractions were immunoblotted with antibody to MAP kinase or were immunoprecipitated with PY20 antibody and assayed for histone H1 kinase activity. As shown in Figure 7, the histone H1 kinase activity was completely separated from the two isoforms of MAP kinase; no other peaks of histone H1 kinase activity were observed. Negligible histone H1 kinase activity was observed in immunoprecipitates from 3Y1 fractions (unpublished data). The effect of 200 nM staurosporine on the histone H1 kinase activity was assessed. Several MAP kinase isoforms have been shown to be resistant to staurosporine at this concentration (Kyriakis and Avruch, 1990; Boulton *et al.*, 1991; Gotoh *et al.*, 1991). In contrast, the histone H1 kinase activity present in SR-3Y1 PY20 eluates was almost completely abolished by 200 nM staurosporine (Table I). These results suggest that the observed histone H1 kinase activity is distinct from previously characterized species of MAP kinase.

Discussion

We have presented an application of anti-phosphotyrosine immunoaffinity chromatography to characterize substrates of the pp60^{v-src} tyrosine kinase. This enrichment step has

been used previously in the generation of monoclonal antibodies against individual tyrosine phosphoproteins (Glenney and Zokas, 1989; Kanner *et al.*, 1990). However, some relevant substrates of pp60^{v-src} might not be present in sufficient quantities to be visualized directly and this low abundance or poor immunogenicity may preclude the generation of antibodies. An additional strategy is the detection of pp60^{v-src} substrates present in anti-phosphotyrosine column eluates by enzymatic assay. This opportunity to study the enzymatic activity of tyrosine phosphoproteins in solution offers an advantage over immune complex assays, because potential distortion of enzyme conformation by the antibody can be avoided. The precedent of serine/threonine kinase activation initiated by tyrosine kinases encouraged us to search for serine/threonine kinases in the anti-phosphotyrosine column eluates derived from v-src-transformed fibroblasts.

By enrichment of tyrosine phosphoproteins with a PY20 anti-phosphotyrosine immunoaffinity resin, we have identified a serine kinase activity that is directly modulated by tyrosine phosphorylation in SR-3Y1 cells. The elevated histone H1 kinase activity in SR-3Y1 PY20 eluates relative to 3Y1 PY20 eluates may be due in part to an increased quantity of kinase in the former preparation, because tyrosine phosphorylation may cause the relevant serine kinase to be more reactive with the PY20 antibody. However, treatment of the kinase preparation with the catalytic domain of the LAR tyrosine phosphatase inactivated the histone H1 kinase and this result indicated that the specific activity of the histone H1 kinase is modulated by tyrosine phosphorylation. It is as yet unclear, however, whether the serine kinase is itself phosphorylated on tyrosine or whether a physically associated tyrosine phosphoprotein modulates the activity of a catalytic subunit.

The histone H1 kinase activity is unlikely to be attributable to known kinases regulated by second messengers, because modulators of these kinases did not significantly affect the kinase activity in PY20 eluates. The catalytic subunit of cAMP-dependent protein kinase has been shown to be modified by pp60^{v-src} (Graziani *et al.*, 1984). However, the histone H1 kinase activity present in PY20 eluates was not significantly inhibited by a peptide inhibitor of cAMP-dependent protein kinase (Table I, JK4 peptide). The pp70 ribosomal S6 kinase and pp90 S6 kinase II are unlikely to be responsible for the observed kinase activity, because these protein kinases do not efficiently phosphorylate histone H1 (Erikson and Maller, 1985; Price *et al.*, 1989). The histone H1 kinase activity is likely to be distinct from previously described MAP kinase isoforms. As shown in Figure 7, the p42 and p44 isoforms of MAP kinase were completely separable from the histone H1 kinase activity that bound to PY20 beads. The observed histone H1 kinase activity was highly sensitive to inhibition by staurosporine and this property distinguishes the histone H1 kinase from characterized species of MAP kinase (Kyriakis and Avruch, 1990; Boulton *et al.*, 1991; Gotoh *et al.*, 1991). Moreover, characterized species of MAP kinase do not efficiently phosphorylate histone H1 (Ahn *et al.*, 1990; Kyriakis and Avruch, 1990; Boulton *et al.*, 1991).

The histone H1 kinase present in PY20 eluates is distinct from p34^{cdc2}. Although the histone H1 kinase activity could bind to p13^{suc1} beads *in vitro*, this kinase activity could be separated from proteins (such as p34^{cdc2}) that were

recognized by anti-PSTAIR antibody. Moreover, tyrosine dephosphorylation of the histone H1 kinase present in SR-3Y1 PY20 eluates resulted in its inactivation, whereas dephosphorylation of tyrosine and threonine residues correlates with an increase in p34^{cdc2} histone H1 kinase activity (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Morla *et al.*, 1989). p34^{cdc2} isolated from v-src-transformed chicken embryo fibroblasts is tyrosine-phosphorylated only on Tyr15 (Krek and Nigg, 1991), the site of negative regulatory phosphorylation and this result suggests that pp60^{v-src} does not activate the p34^{cdc2} kinase by phosphorylation of a novel tyrosine residue. The findings described here indicate that the histone H1 kinase activated in v-src-transformed fibroblasts is distinct from p34^{cdc2}.

Recently, protein kinases related to p34^{cdc2} have been identified (Lehner and O'Farrell, 1990; Elledge and Spottswood, 1991; Paris *et al.*, Tsai *et al.*, 1991). The products of these *cdc2*-related genes, termed *cdk2*, bind to p13^{suc1} (Dunphy *et al.*, 1988; Fang and Newport, 1991; Giordano *et al.*, 1991) and possess inherent histone H1 kinase activity (Fang and Newport, 1991; Tsai *et al.*, 1991). However, the *cdk2* protein also contains the PSTAIR motif. The separation of the observed histone H1 kinase activity from proteins reactive with anti-PSTAIR antibody suggests that this kinase is distinct from the *cdk2* protein.

The results presented here indicate that a serine/threonine-type kinase that phosphorylates histone H1 *in vitro* is activated by tyrosine phosphorylation in v-src-transformed fibroblasts. This kinase may mediate pp60^{v-src}-initiated signal transduction at a position adjacent to this transforming protein. The precise mechanism by which pp60^{v-src} activates this kinase is of particular interest, as is the role of this serine/threonine-type kinase in the normal control of cell proliferation.

Materials and methods

Reagents

Sodium orthovanadate (Na₂VO₄) was from Fisher Scientific and 50 mM stock solutions were prepared as described previously by Kypta *et al.* (1988). Leupeptin, dithiothreitol (DTT), histone H1 and Triton X-100 were from Boehringer Mannheim. Sodium molybdate (Na₂MoO₄), aprotinin, phenylmethylsulfonyl fluoride (PMSF), ovalbumin, phenyl phosphate, casein, myelin basic protein, protamine, 8-bromo-cAMP and 8-bromo-cGMP were from Sigma. All other compounds were reagent grade or better. [γ -³²P]ATP and ¹²⁵I-labeled protein A were from Amersham. Sephacryl S-200, Superose 12 (Prep Grade) and cyanogen bromide-activated Sepharose 4B were from Pharmacia LKB Biotechnology. PY20 antibody conjugated to Affi-Gel (10 mg antibody/ml gel) was generously provided by John Glenney (University of Kentucky, Lexington, KY). The catalytic domain of the LAR tyrosine phosphatase was a gift from Haruo Saito (Harvard Medical School, Boston, MA). MAP-2 purified from pig brain was provided by Tadayo Miyasaka and Alan Saltiel (Parke-Davis Pharmaceuticals, Ann Arbor, MI). Mouse monoclonal antibody against the PSTAIR motif was provided by Yoshitaka Nagahama (National Institute for Basic Biology, Okazaki, Japan). Mouse monoclonal antibody against MAP kinase was from Zymed. Rabbit polyclonal antibody (G8) against *Schizosaccharomyces pombe* p34^{cdc2} was a gift from David Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Bacteria expressing p13^{suc1} were provided by Fred Cross (The Rockefeller University, New York, NY). Calmodulin and the kinase inhibitory peptides JK4, JK77 and BB91 were provided by Angus Nairn and Andrew Czernik (The Rockefeller University, New York, NY).

Cell culture and preparation of extracts

Rat 3Y1 fibroblasts and 3Y1 cells transformed by the Schmidt-Ruppin strain of Rous sarcoma virus (SR-3Y1) (Kawai, 1980) were grown at 37°C in Dulbecco's Eagle Medium containing 5% calf serum (5% DEM). Cells were starved of serum for 12 h prior to lysis. 3Y1-NY72^{ts} is a 3Y1 cell

line transformed with a temperature-sensitive *v-src* variant, which was introduced in a packaging-defective strain of Moloney murine leukemia virus (provided by Irwin Gelman, Mount Sinai School of Medicine, New York, NY). 3Y1–NY72^{ts} cells were maintained at 34°C in 5% DEM. For temperature shift experiments, 3Y1–NY72^{ts} cells were grown in 5% DEM at 39.5°C (non-permissive temperature) for at least 12 h prior to shifting to 34°C (permissive temperature).

Cells grown to near confluency were extracted directly on tissue culture plates with lysis buffer (10 mM Tris, pH 7.2, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 2 mM DTT, 1 mM Na₃VO₄, 0.1 mM Na₂MoO₄, 20 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM PMSF) for 5 min at 4°C (1 ml/100 mm plate). Lysates were clarified by centrifugation (15 000 g for 5 min) and the concentration of protein in the supernatant was measured using a Bio-Rad protein assay kit.

Chromatography

Equivalent amounts of soluble protein from cell lysates were batch incubated on a wrist action shaker with 0.5 ml PY20–Affi-Gel beads (1–2 mg cell lysate protein/ml beads) for 2 h at 4°C. The slurry was then packed into a column and the resin was washed with 40 column vol (20 ml) of wash buffer (lysis buffer without PMSF or leupeptin). A further 1 ml of wash buffer was applied to the column and the eluate was retained. Bound protein was eluted with sequential 1 ml vol of 40 mM phenyl phosphate in wash buffer. In some experiments, eluates were collected into ovalbumin carrier (final concentration 1 mg/ml). However, the presence of ovalbumin was found to have no effect on the recovery of histone H1 kinase activity.

Anion exchange chromatography was carried out at 4°C using a Pharmacia LKB Biotechnology FPLC system. Equal volumes of PY20 eluates were fractionated on a MonoQ HR 5/5 column equilibrated with PY20 column wash buffer. Fractionation was carried out at 1.0 ml/min. The column was washed with 4 ml buffer and then developed with a 20 ml linear gradient of 50 mM–1 M NaCl in wash buffer. Fractions (1.0 ml) were collected in tubes containing ovalbumin as a carrier protein (final concentration 1 mg/ml). For fractionation of whole cell lysates, 1.5 mg of 3Y1 or SR-3Y1 cell protein was applied to the MonoQ column, which was then washed with 10 ml PY20 column wash buffer at 0.5 ml/min. The column was developed with a 20 ml linear gradient of 50–620 mM NaCl at 0.5 ml/min and 0.5 ml fractions were collected. No ovalbumin was present in the collection tubes.

For gel filtration chromatography, 0.25 ml PY20 eluate was applied to a Superose 12 column (0.7 × 50 cm) equilibrated with 20 mM Tris, pH 7.2, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 2 mM DTT, 1 mM Na₃VO₄, 0.1 mM Na₂MoO₄, 1 µg/ml leupeptin and 2 µg/ml aprotinin. The column was developed at a flow rate of 1 ml/h and 0.25 ml fractions were collected.

Immunoprecipitation

PY20 eluates were incubated with G8 antibody against p34^{cdc2} and immune complexes were collected with protein A–Sephacrose beads (Pharmacia LKB Biotechnology). Pellet and supernatant fractions were assayed for histone H1 kinase activity or were immunoblotted with anti-PSTAIR antibody (see below).

MonoQ fractions of whole cell lysates were immunoprecipitated with 1 µg PY20 antibody and immune complexes were collected with protein G–Sephacrose beads (Pharmacia LKB Biotechnology). Immune complexes were washed sequentially with PY20 column wash buffer (three times) and with kinase reaction buffer (20 mM Tris, pH 7.2, 2 mM DTT, 1 mM EGTA, 10 mM MgCl₂ and 200 µM Na₃VO₄). Adsorbed histone H1 kinase activity was assayed as described below.

Immunoblotting

Polyclonal antibody against phosphotyrosine was prepared as described previously (Wang, 1985). Samples were fractionated by SDS–10% PAGE and transferred to a nylon membrane (Immobilon-P, Millipore) in transfer buffer (25 mM Tris, 92 mM glycine, pH 8.6 and 10% methanol). For detection of tyrosine phosphoproteins, the membrane was probed with polyclonal anti-phosphotyrosine antibody followed by ¹²⁵I-labeled protein A. Membranes probed with anti-PSTAIR antibody or antibody to MAP kinase were visualized by ECL (Amersham) according to the manufacturer's instructions.

Kinase assays

Kinase preparations were assayed in a final volume of 50 µl containing 20 mM Tris, pH 7.2, 2 mM DTT, 1 mM EGTA, 10 mM MgCl₂, 0.2 mg/ml histone H1 and 10 µCi [^γ-³²P]ATP (3000 Ci/mmol). 10 µl kinase in solution phase, immunoprecipitated kinase, or kinase adsorbed to p13^{suc1} beads (see below) was assayed for 10 min at 30°C. Reactions were stopped

by the addition of an equal volume of SDS sample buffer containing 50 mM EDTA, followed by boiling for 5 min prior to separation on SDS–12.5% PAGE. Dried gels were autoradiographed and quantitated by densitometry.

Histone H1 kinase activity adsorbed to PY20 antibody was assayed for 15 min at 22°C in the presence of 200 µM Na₃VO₄.

Phosphoamino acid analysis

Extraction of radiolabeled proteins from gel slices, acid hydrolysis and one-dimensional electrophoresis of phosphoamino acids were performed as described previously by Hunter and Sefton (1980).

Tyrosine phosphatase treatment

For tyrosine phosphatase treatment, PY20 eluates were prepared as described above, except that column wash and elution buffers contained no Na₃VO₄ or Na₂MoO₄. 100 µl vol of SR-3Y1 PY20 eluate were incubated with purified domain 1 of the LAR tyrosine phosphatase (final concentration 0.23 mg/ml) in the presence of 10 mM DTT. Reactions were carried out at 30°C for 30 min in the presence or absence of 1 mM Na₃VO₄/1 mM Na₂MoO₄. Phosphatase reactions performed in the absence of phosphatase inhibitor were stopped by the addition of Na₃VO₄ and Na₂MoO₄ to a final concentration of 1 mM each. An aliquot (25 µl) of the sample was used for the determination of histone H1 kinase activity as described above.

Purification of p13^{suc1} and precipitation with p13^{suc1}-coupled beads

Recombinant p13^{suc1} expressed in bacteria was prepared as described previously by Brizuela *et al.* (1987). Briefly, a bacterial cell lysate containing p13^{suc1} was centrifuged at 100 000 g for 1 h and the supernatant was retained. The p13^{suc1} protein was purified to near homogeneity by gel filtration chromatography of this supernatant through a Sephacryl S-200 column. Peak fractions of p13^{suc1} were coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions.

10 µl p13^{suc1}-coupled beads (5 mg protein/ml beads) were combined with 20 µl PY20 eluate in a total volume of 500 µl cell lysis buffer. This mixture was incubated for 1 h at 4°C on a wrist-action shaker. The beads were then washed sequentially with cell lysis buffer (three times) and with kinase reaction buffer (20 mM Tris, pH 7.2, 2 mM DTT, 1 mM EGTA, 10 mM MgCl₂). Histone H1 kinase activity adsorbed to beads was assayed as described above.

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